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The LpxL acyltransferase is required for normal growth and penta-acylation of lipid A in *Burkholderia cenocepacia*

Yasmine Fathy Mohamed [1,2], Mohamad Hamad [3], Ximena P. Ortega [3], and Miguel A. Valvano [1,3]*

[1] Centre for Infection and Immunity, Queen's University Belfast, Belfast, BT9 7BL, United Kingdom, [2] Department of Microbiology and Immunology, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt, [3] Department of Microbiology and Immunology, University of Western Ontario, London, ON, N6A 5C1, Canada.

* For correspondence: E-mail m.valvano@qub.ac.uk; Tel (+44) 28 9097 6025.

Summary

Lipid A anchors the lipopolysaccharide (LPS) to the outer membrane and is usually composed of a hexa-acylated diglucosamine backbone. *Burkholderia cenocepacia*, an opportunistic pathogen, produces a mixture of tetra- and penta-acylated lipid A. "Late" acyltransferases add secondary acyl chains to lipid A after the incorporation of four primary acyl chains to the diglucosamine backbone. Here, we report that *B. cenocepacia* has only one late acyltransferase, LpxL (BCAL0508), which adds a myristoyl chain to the 2' position of lipid A resulting in penta-acylated lipid A. We also identified PagL (BCAL0788), which acts as an outer membrane lipase by removing the primary β-hydroxymyristate (3-OH-C14:0) chain at the 3 position, leading to tetra-acylated lipid A. Unlike PagL, LpxL depletion caused reduced cell growth and defects in cell morphology, both of which were suppressed by overexpressing the LPS flippase MsbA (BCAL2408), suggesting that lipid A molecules lacking the fifth acyl chain contributed by LpxL are not good substrates for the flippase. We also show that intracellular *B. cenocepacia* within macrophages produced more penta-acylated lipid A, suggesting lipid A penta-acylation in *B. cenocepacia* is required not only for bacterial growth and morphology but also for adaptation to intracellular lifestyle.

Introduction

Lipopolysaccharide (LPS), the chief constituent of the surface-exposed leaflet of the Gram-negative bacterial outer membrane, consists of discrete structural regions including lipid A, core oligosaccharide (core), and in many bacteria the repeating O-antigen units (Nikaido, 2003). The lipid A is a glycolipid that anchors LPS to the outer membrane; its structure and that of the inner portion of the core are highly conserved (Raetz *et al.*, 2007, Whitfield and Trent, 2014). Lipid A-core biogenesis starts at the cytoplasmic face of the inner membrane and then the molecule is transported across the membrane to the periplasmic side. LPS export to the outer membrane's external leaflet occurs through LPS transport proteins (Lpt), which form a multi-protein complex that links the inner and outer membranes and consumes energy from ATP hydrolysis (Okuda *et al.*, 2016).

LPS, particularly lipid A, is one of the most characteristic microorganism-associated molecular patterns activating the host innate immune system. Lipid A is detected by the Toll-like receptor 4 (TLR4) in complex with the myeloid differentiation factor 2 (MD2), both of which are on the cell surface of immune cells like macrophages and monocytes (Beutler, 2002, Di Lorenzo *et al.*, 2015, Park and Lee, 2013). Binding of lipid A to TLR4–MD2 triggers the release of pro-inflammatory cytokines resulting in an inflammatory response that eventually clears the infection. This inflammatory response can be highly robust and its deregulation leads to potentially fatal consequences especially in cases of sepsis (Bryant *et al.*, 2010).

Bacteria modify and remodel the basic lipid A backbone in response to environmental cues. These modifications become critical for bacteria to infect and survive in the host (Raetz *et al.*, 2007). For example, *Escherichia coli* lipid A, with six acyl chains and 2 phosphate groups is the strongest known TLR4 agonist; however, the tetra-acylated form is an antagonist that blocks the TLR4 signalling pathway (Needham and Trent, 2013). Other modifications of the lipid A can either render LPS unrecognizable as a microorganism-associated molecular pattern or impart bacterial resistance to immune effectors, such as

antimicrobial peptides and antibiotics. This can be accomplished through addition of positively charged chemical groups to the lipid A phosphates, such as phosphoethanolamine (Lee *et al.*, 2004, Kim *et al.*, 2006), 4-L-amino-4-deoxyarabinose (L-Ara4N) (Trent *et al.*, 2001), galactosamine (Wang *et al.*, 2009) and glucosamine (Marr *et al.*, 2008). These substitutions reduce the overall negative charge of the bacterial surface promoting resistance to antimicrobial peptides such as polymyxin B. The degree of lipid A acylation and phosphorylation is also important for lipid A recognition by TLR4-MD2 complex and for membrane packing, which maintains the stability of the outer membrane and reduces the diffusion of hydrophobic molecules. Therefore, changes in the number and properties of acyl chains play an important role in lipid A adaptive modifications (Needham and Trent, 2013).

Gram-negative pathogens have evolved several adaptive LPS modification strategies (Needham and Trent, 2013, Raetz *et al.*, 2007). Lipid A remodelling can occur either during or after trafficking to the cell surface. In general, the modification enzymes are not essential for bacterial viability *in vitro* but they can be critical during infection (Needham and Trent, 2013).

Burkholderia cenocepacia is an opportunistic Gram-negative pathogen that has become a health threat to patients with cystic fibrosis (Mahenthiralingam *et al.*, 2008, Vandamme and Dawyndt, 2011). The *B. cenocepacia* lipid A consists of two linked glucosamine residues that contain phosphodiester-linked L-Ara4N residues (Silipo *et al.*, 2005). Unlike most bacteria, the L-Ara4N residues are essential for *B. cenocepacia* viability and LPS export to the outer membrane (Hamad *et al.*, 2012, Ortega *et al.*, 2007). A disaccharide made of 3-*deoxy*-D-*manno*-oct-2-ulosonic acid (Kdo) and D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko) is attached to the *B. cenocepacia* lipid A, instead of the prototypical Kdo-Kdo disaccharide in other bacteria (Silipo *et al.*, 2005). The Ko residue is also glycosylated by L-Ara4N (Silipo *et al.*, 2005, De Soyza *et al.*, 2008). The rest of the core is attached to the Kdo residue of the lipid A-Kdo-Ko-L-Ara4N molecule. Some *B. cenocepacia* isolates also contain an O-antigen polysaccharide ligated to core and composed of a repeating trisaccharide unit made of two residues of *N*-acetyl galactosamine and one rhamnose residue (Ortega *et al.*, 2005, Ortega

et al., 2009).

Classical lipid A synthesis requires a nine-step enzymatic machinery known as the Raetz pathway (Whitfield and Trent, 2014). The *B. cenocepacia* genome contains homologs of genes encoding eight of the nine canonical enzymes required for the lipid A biosynthesis in *E. coli* (Fig. 1). The Raetz pathway starts by the acylation of the sugar nucleotide; uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) by LpxA (BCAL2079) (Anderson *et al.*, 1985, Anderson and Raetz, 1987). This is followed by five enzymatic steps catalysed by LpxC (BCAL3455), LpxD (BCAL2081), LpxH (BCAL2154), LpxB (BCAL2078), LpxK (BCAL2759) to produce the lipid IV_A molecule (Fig. 1A) (Jackman *et al.*, 1999, Kelly *et al.*, 1993, Babinski *et al.*, 2002a, Babinski *et al.*, 2002b, Crowell *et al.*, 1986, Garrett *et al.*, 1997). Kdo-Kdo residues are then incorporated to lipid IV_A by WaaA (BCAL3310), which uses cytidine monophosphate (CMP)-Kdo as the donor substrate (Fig. 1B) (Clementz and Raetz, 1991). In *Burkholderia*, the distal Kdo residue is converted to Ko by a dioxygenase (Chung and Raetz, 2011, Chung *et al.*, 2014) (KdoO; BCAL3311; Hamad and Valvano, unpublished; Fig. 1B). In *E. coli*, addition of secondary acyl chains takes place after the Kdo addition (Reynolds and Raetz, 2009) and these steps are performed by two membrane-bound "late" acyltransferases, LpxL and LpxM. LpxL transfers a lauroyl (C12:0) group to the 2' position, while LpxM adds a myristoyl (C14:0) group to the 3' position (Clementz *et al.*, 1996, Clementz *et al.*, 1997). *B. cenocepacia* has only one extra C14:0 acyl chain added to the 2' position, resulting in penta-acylated lipid A (Fig. 1B) (Silipo *et al.*, 2005).

After completion of the incorporation of acyl chains, the lipid A-core moiety is flipped across the inner membrane by the LPS flippase (MsbA), an ABC transporter, and the O-polysaccharide formed by an independent pathway is ligated to a terminal core residue (Doerrler *et al.*, 2001, Polissi and Georgopoulos, 1996). MsbA is specific for the degree of lipid A acylation, thus ensuring that only lipid A species with the appropriate number of acyl chains are translocated (Doerrler and Raetz, 2002). LPS is an important virulence factor in *B. cenocepacia* (Di Lorenzo *et al.*, 2015, Khodai-Kalaki *et al.*, 2015, Kotrange *et al.*, 2011, Ortega *et al.*, 2009). However, the contribution of the lipid A biosynthetic machinery and

modifications to the pathogenicity and robustness of the bacterium has not been systematically investigated (Maldonado *et al.*, 2016). Unlike *E. coli* and *Salmonella enterica*, lipid A from *B. cenocepacia* and other *Burkholderia* species consists of a mixture of tetra and penta-acylated species containing one secondary acyl chain (Fig. 1B) (Silipo *et al.*, 2005, Silipo *et al.*, 2007). The molecular basis for this distribution of acyl chains is unknown. In this study, we established the presence of only one "late" acyltransferase, $LpxL_{Bc}$, which is responsible for the addition of the fifth acyl chain to *Burkholderia* lipid A at the 2' position. We also identified the *B. cenocepacia* PagL lipase that catalyses the 3-O-deacylation at position 3, resulting in tetra-acylated lipid A. Further, we show that $LpxL_{Bc}$ is required for normal growth and cell morphology of *B. cenocepacia*, which in the absence of $LpxL_{Bc}$ expression could be regained by overexpressing the MsbA LPS flippase. We also found that *B. cenocepacia* has higher preference towards formation of penta-acylated lipid A in infected macrophages, suggesting that penta-acylation of lipid A is important for *B. cenocepacia* adaptation to the intracellular lifestyle.

Results

LpxL$_{Bc}$ is required for normal growth and cell morphology of B. cenocepacia

We searched the *B. cenocepacia* genome for LpxM and LpxL acyltransferase orthologues that add secondary acyl chains to the lipid A-Kdo-Ko. BCAL0508 was the only gene identified in *B. cenocepacia* encoding a predicted "late" lipid A acyltransferase. BCAL0508 had 30% amino acid identity to *E. coli* LpxM and 34% identity to LpxL. Because the extra acyl chain is incorporated at the 2' position of the lipid A (Fig. 1B), which is the location of the acyl chain added to the lipid A by the LpxL orthologues in *E. coli* and *S. enterica*, we renamed BCAL0508 as LpxL$_{Bc}$. Repeated attempts to delete the *lpxL*$_{Bc}$ gene from strain K56-2 using the targeted, unmarked deletion method (Flannagan *et al.*, 2008, Aubert *et al.*, 2014) consistently failed, suggesting this gene could be required for *B. cenocepacia* viability. We constructed a conditional mutant by placing *lpxL*$_{Bc}$ under the control of a rhamnose-inducible promoter (Cardona *et al.*, 2006). The resulting strain, K56-2(*P$_{rha}$*::*lpxL*$_{Bc}$) (YFM51 in Table 1), had reduced growth under the non-permissive condition (0.2% glucose; Fig. 2A). This was confirmed by a rhamnose depletion assay in minimal medium monitoring growth under permissive and non-permissive conditions for up to 20 h. K56-2(*P$_{rha}$*::*lpxL*$_{Bc}$) grew poorly in glucose showing some growth recovery at later time points (Fig. 2B), suggesting that LpxL depletion affects growth rate. Delayed growth recovery under the non-permissive condition could be due to incomplete halt of *lpxL*$_{Bc}$ gene transcription. Individual bacterial cells were examined by phase contrast microscopy, differential interference contrast (DIC) microscopy, and transmission electron microscopy. In the presence of rhamnose, K56-2(*P$_{rha}$*::*lpxL*$_{Bc}$) cells formed short rods at all time points (Fig 2C). In contrast, at late time points virtually all K56-2(*P$_{rha}$*::*lpxL*$_{Bc}$) cells from cultures in 0.2% glucose displayed elongated morphology forming isolated diplobacilli and also multicellular chains, indicative of cell division defects (Fig. 2C, arrows). Fluorescence staining with syto-9, a DNA intercalating dye, confirmed that cells contained 2 nucleoids and also some cells showed deformed nucleoids (Fig. 2C, arrowheads). Ultrastructural analysis of rhamnose-depleted cultures by

transmission electron microscopy revealed incomplete cell divisions, accumulation of intramembranous structures, and the presence of empty sacculi (Fig. 3). These morphological defects resemble those observed when LPS export to the outer membrane is halted (Wu *et al.*, 2006), as we have previously demonstrated in conditional mutants defective in L-Ara4N synthesis (Ortega *et al.*, 2007), which have an LPS export defect (Hamad *et al.*, 2012). Together, our results support the notion that $LpxL_{Bc}$ is required for the normal growth and cell morphology of *B. cenocepacia*.

Conditional suppression of $LpxL_{Bc}$ expression leads to a mixture of tri- and tetra-acylated lipid A

Lipid A analysis was carried out on $LpxL_{Bc}$-depleted cultures in K56-2(P_{rha}::*lpxL*$_{Bc}$) using negative ion matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. The *B. cenocepacia* lipid A exhibited a complex fragmentation pattern of molecular ion peaks representing tetra and penta-acylated species (see all the assignments in Table 2), as previously described (Sturiale *et al.*, 2011). Also, the fragmentation pattern was characteristic for the cleavage of the labile bond between Kdo and lipid A (Sturiale *et al.*, 2005). Under the permissive condition (0.2% rhamnose), the MALDI-TOF spectrum of lipid A obtained from K56-2(P_{rha}::*lpxL*$_{Bc}$) (Fig. 4A) was identical to that of the parental K56-2 strain (Fig. 4C) and the previously characterized spectrum of the K56-2 *wabR* mutant (Sturiale *et al.*, 2011, Ortega *et al.*, 2009). Therefore, neither the presence of rhamnose in the growth medium nor the insertion of the rhamnose inducible promoter in the bacterial genome had any detectable effect on the lipid A profiles. In all cases, the MALDI-TOF spectrum contains a characteristic ion peak at m/z 1444.7 corresponding to tetra-acylated, bisphosphorylated glucosamine disaccharide (Fig. 4A, left spectrum, and Table 2) (Hamad *et al.*, 2012, Sturiale *et al.*, 2011). This ion represents a lipid A molecule containing two C16:0(3-OH) chains, one C14:0(3-OH) and one secondary C14:0 chain (Fig. 4B, left, and Table 2). This lipid A molecule lacks the primary β-hydroxymyristate (3-OH-C14:0) chain at the 3 position (see below). There was also a peak at m/z 1576.8 representing the addition of

L-Ara4N ($\Delta m/z$ = +131 to the peak at m/z 1444.7). The ion peaks at m/z 1364.7 and m/z 1495.9 correspond to mono-phosphorylated versions ($\Delta m/z$ = -80 to 1444.7 and 1576.8 ion peaks, respectively; Table 2). Peaks at m/z 1671 and 1803.1 were attributed to penta-acylated, bisphosphorylated lipid A species having an additional myristoyl chain [14:0(3-OH)] ($\Delta m/z$ = +226 to the peaks at m/z 1444.7 and 1576.8, respectively; Table 2). The peak at m/z 1707.9 represents a tetra-acylated, bisphosphorylated lipid A species with 2 L-Ara4N residues (Fig. 4A, and Table 2).

In contrast, under the non-permissive condition (0.2% glucose), each of the former peaks appeared, but with $\Delta m/z$= -210 that corresponds to the loss of the secondary C14:0 chain attached to the C16:0(3-OH) chain at the 2' position (Fig. 4A, right spectrum, 4B). Therefore, we conclude that lipid A produced under the non-permissive condition consists of a mixture of tri and tetra-acylated lipid A species. Indeed, the ion peak at m/z 1234.6 corresponds to a tri-acylated bisphosphorylated diglucosamine and that at m/z 1366.7 corresponds to tri-acylated bisphosphorylated diglucosamine with an extra L-Ara4N (Table 2). The ion peaks at m/z 1154.6 and 1285.7 denote the mono-phosphorylated versions of the previous peaks. The two peaks at m/z 1460.9 and 1593.1 correspond to the tetra-acylated derivatives represented by the peaks at m/z 1234.5 and 1366.7, respectively, which have an extra C14:0(3-OH) acyl chain ($\Delta m/z$ = +226; Table 2). Relatively small ion peaks of penta-acylated lipid A appeared at m/z 1671.2 and 1803.2 (Fig. 1A and Table 2), likely due to incomplete depletion of *lpxL*$_{Bc}$ gene transcription under non-permissive conditions. Together, the MALDI-TOF mass spectrometry analysis supports the notion that LpxL$_{Bc}$ is the late acyltransferase responsible for adding the fifth acyl chain to the *B. cenocepacia* lipid A.

Identification of PagL, a lipase responsible for the hypo-acylated lipid A species in B. cenocepacia

Irrespective of the presence or absence of the LpxL$_{Bc}$ mediated secondary acyl chain, the tetra-acylated and tri-acylated forms of *B. cenocepacia* K56-2 lipid A under permissive and non-permissive conditions, respectively, corresponded to the loss of the primary β-

hydroxymyristate (3-OH-C14:0) chain, which was previously shown to be at the 3 position of the lipid A glucosamine I by negative-ion MALDI-TOF/TOF fragmentation (Sturiale *et al.*, 2011) (Fig. 4B). This suggests a 3-*O*-deacylase activity in *B. cenocepacia*. Scanning the genome of *B. cenocepacia*, we identified BCAL0778, encoding a putative PagL 3-*O*-deacylase orthologue, which could be responsible for the removal of the lipid A acyl chain at the 3 position; this gene was renamed *pagL*$_{Bc}$. We constructed a *pagL*$_{Bc}$ deletion mutant and examined its lipid A profile by MALDI-TOF mass spectrometry. Fig. 4C shows that loss of *pagL*$_{Bc}$ results in penta-acylated lipid A and the concomitant loss of the ion peaks corresponding to tetra-acylated molecular species, except for the ion peak at m/z 1444.7 corresponding to native tetra-acylated, bisphosphorylated glucosamine disaccharide (Table 2). Despite MALDI-TOF is not quantitative, in relative terms the 1444.7 ion peak is relatively small in Δ*pagL*$_{Bc}$ compared to wild type (Fig. 4D), suggesting the possibility of another 3-*O*-deacylase activity in *B. cenocepacia* that is distinct of PagL.

MsbA$_{Bc}$ *complements the growth defect and cell morphology phenotypes caused by LpxL*$_{Bc}$ *depletion*

The reduced viability and the morphological cell defects under LpxL$_{Bc}$ depletion suggest a block in the export of lipid A-core. MsbA is the LPS flippase that transports the lipid A-core across the inner membrane (Polissi and Georgopoulos, 1996). In contrast to penta- and hexa-acylated lipid A species, tetra-acylated lipid A is considered a poor substrate for MsbA, indicating that this protein provides a quality control ensuring the export of appropriate lipid A species (Whitfield and Trent, 2014). BCAL2408 is the putative MsbA of *B. cenocepacia* and it was herein renamed as MsbA$_{Bc}$. Overexpressing MsbA$_{Bc}$ in K56-2 (*P*$_{rha}$::*lpxL*$_{Bc}$) restored the growth defect associated with the conditional mutant under non-permissive condition (Fig. 5A and C). Also, MsbA$_{Bc}$ overexpressed under the non-permissive condition restored cell morphology to normal rod-shape bacterial cells (Fig. 5B). As expected, overexpression of MsbA did not affect the lipid A profile. Indeed, the molecular ion peaks detected by negative ion MALDI-TOF mass spectrometry in K56-2 (*P*$_{rha}$::*lpxL*$_{Bc}$) overexpressing MsbA$_{Bc}$

under the non-permissive condition consisted of a mixture of tri- and tetra-acylated lipid A (Fig. 5D and Table 2). Therefore, we conclude that $MsbA_{Bc}$ overexpression increases the transport of the tri and tetra-acylated lipid A species across the inner membrane, thus alleviating the growth and morphology defects associated to a halt in lipid A-core export under $LpxL_{Bc}$ depletion.

$LpxL_{Bc}$ complements the lipid A defect in E. coli ΔlpxL *but not* ΔlpxM *mutants*

To confirm the functional assignment of BCAL0508 as $LpxL_{Bc}$ we performed complementation experiments in Δ*lpxL* and Δ*lpxM E. coli* strains. For this purpose, *$lpxL_{Bc}$* was cloned in pEXT20 under the control of the β-galactosidase promoter. The recombinant plasmid was introduced into the *E. coli* mutants and protein expression was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). MALDI-TOF mass spectrometry of lipid A of the parental *E. coli* strain W3110 showed a molecular ion peak at m/z 1797.13, which corresponds to hexa-acylated bisphosphorylated diglucosamine and it is the most abundant ion peak of *E. coli* lipid A containing four 3-hydroxymyristoyl [C14:0(3-OH)] chains, one myristoyl (C14:0) chain, and one lauroyl (C12:0) chain (Fig. 7). Another ion peak appeared at m/z 1360.52, which corresponds to tetra-acylated bisphosphorylated diglucosamine consisting of one lauroyl and three 3-hydroxymyristoyl chains. These data agree with the established structure of the *E. coli* K-12 lipid A (Raetz and Whitfield, 2002). In *E. coli*, late acyltransferase enzymes LpxL and LpxM add the fifth and sixth acyl chains, respectively, to the lipid IV_A-Kdo_2 precursor (Whitfield and Trent, 2014). LpxL adds a lauroyl (C12:0) chain to the 2' position of the diglucosamine while LpxM subsequently adds a myristoyl (C14:0) chain onto the 3-hydroxymyristoyl [C14:0(3-OH)] chain at the 3' position of the diglucosamine (Clementz *et al.*, 1996, Clementz *et al.*, 1997). The MALDI-TOF mass spectrum of the lipid A from *E. coli* Δ*lpxL* shows the absence of a lauroyl chain corresponding to Δ*m/z*= -182 (Fig. 7), resulting in the appearance of ion peaks at m/z 1615.88 and 1534.14 (penta-acylated), m/z 1404.59 and 1324.60 (tetra-acylated) and m/z 1178.29 and 1098.31 (tri-acylated). These peaks correspond to bisphosphorylated and monophosphorylated lipid A species,

respectively. In contrast, the MALDI-TOF mass spectrum of the lipid A of the complemented strain (Δ*lpxL*$_{Ec}$ + LpxL$_{Bc}$) shows an additional ion peak at m/z 1825.18 (Fig. 7), which likely corresponds to hexa-acylated lipid A containing a myristoyl (C14:0) chain at the 2' position of the diglucosamine backbone. This differs from the normally added lauroyl (C12:0) chain in the parental *E. coli* strain W3110. We conclude that although LpxL$_{Bc}$ is functional in *E. coli* it has a strict specificity for a myristoyl (C14:0) fatty acid. A similar observation was reported for *Francisella tularensis* (McLendon *et al.*, 2007), suggesting that LpxL orthologues are highly specific for the fatty acid substrates in their original backgrounds.

The MALDI-TOF mass spectrum of the lipid A from the Δ*lpxM* mutant lacked a myristoyl chain, corresponding to a Δ*m/z*= -210 (Fig. 7). The ion peaks at m/z 1586.91 and 1507 (penta-acylated) and m/z 1360.58 and 1280.58 (tetra-acylated) correspond to bisphosphorylated and monophosphorylated structures, respectively (Rubin *et al.*, 2014). In contrast to the results with Δ*lpxL*, the complemented strain (Δ*lpxM*$_{Ec}$ + LpxL$_{Bc}$) and Δ*lpxM* showed exactly the same lipid A MALDI-TOF profile (Fig. 7). Together, the complementation experiments in Δ*lpxL* and Δ*lpxM E. coli* mutants indicate LpxL$_{Bc}$ functions in a similar manner to its *E. coli* orthologue by adding an acyl chain at the 2' position of the lipid A diglucosamine backbone.

Intracellular B. cenocepacia *show higher preference towards the formation of penta-acylated lipid A that correlates with increased* lpxL$_{Bc}$ *gene transcription*

Lipid A modifications have important implications for host-pathogen interactions (Needham and Trent, 2013). *B. cenocepacia* is an opportunistic pathogen that survives and replicate in macrophages (Valvano, 2015). Hence, we investigated whether lipid A modifications occur in *B. cenocepacia* after infection in RAW 264.7 murine macrophages. To increase the number of intracellular bacteria and at the same time eliminate more readily the extracellular bacteria during the infection experiment, we employed *B. cenocepacia* mutants lacking O-antigen (Saldías *et al.*, 2009), which are also sensitive to gentamicin (Hamad *et al.*, 2010). These strains were MH1J, a derivative of the naturally O-antigen defective strain J2315 with

a mutation in a gentamicin efflux pump, and MH1K ΔBCAL3119-BCAL3131, which has a deletion removing most of the O-antigen genes (Hanuszkiewicz *et al.*, 2014). While both strains exhibit identical lipid A MALDI-TOF mass spectra as that of K56-2 when grown in LB (Fig. 7A), the fragmentation pattern of the lipid A isolated from infected macrophages shows a clear shift towards penta-acylated lipid A (Fig. 7C and D, and Table 2). Particularly, the ion peak 1933, which corresponds to penta-acylated bisphosphorylated lipid A with 2 Ara4N residues (Table 2) and rarely appeared under *in vitro* growth conditions (LB), was very obvious upon macrophage infection. From these experiments, we conclude that intracellular environment in macrophages exerts a selective pressure on *B. cenocepacia* bacterial cells to predominantly produce penta-acylated lipid A.

We hypothesised that the hyperabundance of penta-acylated lipid A upon macrophage infection could reflect either increased expression of $lpxL_{Bc}$ or reduced expression of $pagL_{Bc}$. Therefore, we compared the expression levels of both genes by qRT-PCR in intracellular bacteria with their expression levels in bacterial cells grown in DMEM. The results, which were normalized using two standard genes (BCAS0175 and *gyrB*), indicate 3.3-fold and 3.6-fold higher $lpxL_{Bc}$ and $pagL_{Bc}$ expression levels in macrophages compared to the DMEM control. These differences, relative to the standard genes, were significant with a p value < 0.05. We conclude from these data that the higher abundance of penta-acylated lipid A species in intracellular bacteria cannot be explained by a simple differential expression of $lpxL_{Bc}$ and $pagL_{Bc}$ and is likely due to a more complex pattern of regulation of LPS synthesis.

Discussion

Lipid A is a key component of the bacterial LPS that engages the host innate immune system *via* interactions with the TLR4-MD2 complex. The number and distribution of acyl chains in the lipid A, together with the phosphate groups in the diglucosamine backbone, influence the interaction of lipid A with the TLR4-MD2 complex (Schromm *et al.*, 2000). Generally, this complex is strongly activated by hexa-acylated lipid A and weakly activated by hypoacylated lipid A, as the sixth acyl chain bulges from MD2 binding pocket promoting dimerization of the TLR4-MD2 complex (Seydel *et al.*, 2000, Park *et al.*, 2009, Maeshima and Fernandez, 2013). However, *B. cenocepacia* LPS can strongly activate human TLR4-MD2 even though its lipid A possesses a mixture of tetra- and penta-acylated forms (De Soyza *et al.*, 2004, Bamford *et al.*, 2007, Hollaus *et al.*, 2015, Silipo *et al.*, 2007, Di Lorenzo *et al.*, 2015).

In this study, we elucidated the molecular basis of the tetra- and penta-acylation of the *B. cenocepacia* lipid A. We demonstrated that although the lipid A biosynthetic genes are conserved (Whitfield and Trent, 2014), there is only a single orthologue of the late acyltransferase genes, which corresponds to *lpxL*$_{Bc}$. From our experiments using MALDI-TOF mass spectrometry combined with previous structural work by others and us (Hamad *et al.*, 2012, Sturiale *et al.*, 2011) we conclude that LpxL$_{Bc}$ catalyses the addition of a secondary myristoyl chain to the primary acyl chain at the 2' position of the lipid A. This modification results in the formation of a penta-acylated lipid A. Complementation experiments in *E. coli* expressing *lpxL*$_{Bc}$ demonstrated this gene encodes an acyltransferase with specificity for myristoyl chains. Indeed, LpxL$_{Bc}$ adds a myristoyl chain rather than a lauroyl chain to the Lipid A of *E. coli* Δ*lpxL* but not Δ*lpxM*. However, the relative ion intensities of the fully hexa-acylated species in the complemented strain were lower than those of the incomplete acylated forms, which reflect a better functionality of *lpxL*$_{Bc}$ in the normal *B. cenocepacia* background. These experiments confirmed LpxL$_{Bc}$ acts as a myristoyl transferase.

Failure to delete *lpxL*$_{Bc}$ made us believe this gene is essential for bacterial viability. However, using a conditional mutagenesis expression strategy, we showed that depletion of LpxL$_{Bc}$ expression does not cause loss of viability. In contrast, LpxL$_{Bc}$ depleted bacterial cells exhibited abnormal cell architecture, strongly suggesting defects in cell division. Further, the LpxL$_{Bc}$ depletion experiments demonstrated small amounts of tetra- and penta-acylated lipid A detectable by negative ion MALD-TOF, suggesting that repression of the rhamnose inducible promoter controlling *lpxL*$_{Bc}$ may be only partial and explaining why a clean *lpxL*$_{Bc}$ gene deletion could not be obtained. We hypothesized that reduced bacterial growth and the cell division defects under non-permissive conditions for full *lpxL*$_{Bc}$ expression were due to secondary effects caused by a block in the lipid A export across the membrane. This agrees with the notion that tri and tetra-acylated lipid A forms are poor substrates for the MsbA lipid A flippase (Zhou *et al.*, 1998). Indeed, overexpression of MsbA$_{Bc}$ in the *P*$_{rha}$::*lpxL*$_{Bc}$ strain under the non-permissive condition alleviated the lethal phenotype and restored normal cell morphology despite these cells continue to produce tri-and tetra-acylated lipid A. These observations are similar to a previous report demonstrating that extra copies of the cloned *msbA* gene from *E. coli* restored the ability of *htrB* mutants to grow at 42 °C (non-permissive condition) without increasing the extent of lipid A acylation and with a concomitant transport of hypoacylated lipid A species to the outer membrane (Zhou *et al.*, 1998). The preferred lipid A species for MsbA transport in *B. cenocepacia* has not been investigated, and it should also be considered that the tetra-acylated lipid A species from *E. coli* and *B. cenocepacia* have different acyl chain distributions. In any case, our results suggest a preference of MsbA$_{Bc}$ for penta-acylated lipid A species, which were absent in bacterial cells depleted from LpxL$_{Bc}$.

The production of tri- and tetra-acylated forms in *B. cenocepacia P*$_{rha}$::*lpxL*$_{Bc}$ subjected to rhamnose depletion was due in both cases to the loss of the primary myristoyl chain at position 3 of the diglucosamine backbone. This is the same position to which a myristoyl chain is attached in penta-acylated lipid A of other K56-2 strains, but absent in tetra-acylated forms (Di Lorenzo *et al.*, 2015, Hamad *et al.*, 2012, Sturiale *et al.*, 2011). Together, these

observations suggest the presence of a 3-*O*-deacylase activity in *B. cenocepacia*. Lipases that remove acyl chains in the lipid A are typically outer membrane proteins, as these modifications take place after the flipping of lipid A to the periplasmic side of the membrane. In this work, we identified *pagL*, a gene encoding a predicted outer membrane protein with strong similarity to the *E. coli* PagL. Deletion of this gene resulted in the loss of all tetra-acylated lipid A species, except for a reduced the ion peak at m/z 1444. This ion peak corresponds to tetra-acylated, bisphosphorylated lipid A, and its presence suggests the possibility of a second, possibly minor 3-OH deacylase in *B. cenocepacia*, which is under investigation in our laboratory.

Lipid A acylation contributes to LPS toxicity. Lipid A can be modified in response to environmental stress conditions resulting in increased antimicrobial resistance and evasion of recognition by the innate immune system (Llobet *et al.*, 2015, Needham and Trent, 2013). We observed that intracellular *B. cenocepacia* within macrophages produces more penta-acylated lipid A species suggesting that penta-acylated lipid A is important for survival of *B. cenocepacia* inside macrophages. However, the formation of penta-acylated lipid A could not be directly correlated with differential regulation of *lpxL* and or *pagL* since both genes were more expressed intracellularly than in bacterial culture. We have previously shown that intracellular gene expression of *B. cenocepacia* in macrophages is complex and likely involves many gene regulatory cascades (Tolman and Valvano, 2012). The preference of penta-acylated lipid A species in macrophages may increase the proinflammatory nature of the intracellular infection. This agrees with the suggestion that *B. cenocepacia* lipid A activates TLR4 through the interaction of the fifth acyl chain with TLR4 by the aid of longer acyl chains and L-Ara4N residues, which in turn evokes a robust proinflammatory response (Di Lorenzo *et al.*, 2015). A similar situation was also observed in *Haemophilus influenzae* (Swords *et al.*, 2002) and *F. tularensis* (McLendon *et al.*, 2007) in which the expression of the *lpxL* gene increases during infection of human airway epithelial cells and macrophages, respectively compared to *in vitro* growth conditions. *Pseudomonas aeruginosa* strains can also regulate the degree of lipid A acylation in response to environmental conditions as they

normally possess penta-acylated lipid A. However, they can form hexa-acylated lipid A during infection in cystic fibrosis patients eliciting a more robust inflammatory response and possibly increasing the level of membrane packing and the bacterial resistance to antimicrobial peptides (Hajjar *et al.*, 2002).

In conclusion, this work reveals two critical enzymes that control the number of acyl chains of lipid A in *B. cenocepacia*. Elucidation of the lipid A biosynthesis and its remodelling in this group of bacteria could provide novel insights in the pathogenesis of *B. cenocepacia* and other *Burkholderia* species. Future studies are required to address the regulatory mechanism of lipid A synthesis and modifications in *B. cenocepacia* under various conditions including the intracellular environment.

Experimental Procedures

Strains and growth conditions

Bacterial strains and plasmids used in this study are described in Table 1. Bacteria were cultured in Luria-Broth (LB) medium or M9 medium at 37°C. Unless indicated otherwise antibiotics used (final concentrations) were: trimethoprim (50 µg ml^{-1} for *E. coli* and 100 µg ml^{-1} for *B. cenocepacia*), tetracycline (30 µg ml^{-1} for *E. coli* and 100 µg ml^{-1} for *B. cenocepacia*), kanamycin (40 µg ml^{-1} to maintain the helper plasmid pRK2013 in *E. coli*), and gentamicin (50 µg ml^{-1} to counter select against the *E. coli* donor and helper strains in triparental matings).

Recombinant DNA techniques

Restriction enzymes were obtained from New England Bio labs (NEB). T4 DNA ligase and alkaline phosphatase were obtained from Roche Diagnostics. DNA amplifications by PCR were done with *Taq* or HotStar HiFidelity DNA polymerases (Qiagen), and DNA sequencing was performed at GATC Biotech (London, UK). Conjugations were performed by triparental mating (Craig *et al.*, 1989) with the pRK2013 helper plasmid (Figurski and Helinski, 1979). Deletion mutagenesis was performed as previously described (Flannagan *et al.*, 2008, Aubert *et al.*, 2014). The mutagenic plasmid pMH481, for the construction of the *pagL* (BCAL0788) deletion mutant, carried upstream and downstream sequences of *pagL*, which were obtained by PCR amplification with primer pairs 4740/4741 and 4728/4729 (Table 2). These amplicons were digested with SspI/ClaI and ClaI/XbaI, respectively, and triple-ligated into pGPI-SceI-2 (Hamad *et al.*, 2012) between SspI and XbaI sites.

Conditional mutagenesis

Conditional mutants were constructed as described previously (Mohamed and Valvano, 2014, Ortega *et al.*, 2007). A 300-bp fragment of the upstream region of the *lpxL*$_{Bc}$ gene (BCAL0508) was amplified and cloned into pSC200 behind the plasmid-borne rhamnose

promoter. Transformation was carried out in *E. coli* GT115 competent cells by the calcium chloride method (Cohen *et al.*, 1972). Transformants were selected on LB agar plates containing 50 µg trimethoprim ml^{-1}. Plasmids were mobilized into *B. cenocepacia* K56-2, and exconjugants were isolated on LB agar plates supplemented with 100 µg trimethoprim ml^{-1}, 200 µg ampicillin ml^{-1}, 20 µg polymyxin B ml^{-1} and 0.2% rhamnose. This experiment resulted in the isolation of the conditional mutant strain YFM51 (P_{rha}::*lpxL*$_{Bc}$; Table1).

Complementation experiments

We constructed the plasmid pYEM66 (pEXT20-*lpxL*$_{Bc}$), in which *lpxL* (BCAL0508) gene expression was placed under the control of the *lac* promoter using primers Q868 and Q867 (Table S1). The plasmid was introduced into *E. coli* Δ*lpxL* and Δ*lpxM* mutants (Table 1) by electroporation and transformants were selected on LB agar plates containing 50 µg kanamycin ml^{-1} and 100 µg ampicillin ml^{-1}. A plasmid constitutively expressing MsbA$_{Bc}$ (pYM40) was constructed by amplifying the *msbA* gene (BCAL2408) with primers Q714 and Q715 (Table S1). Amplicons were digested with *Nde*I-*Xba*I and cloned into a similarly digested pDA12, resulting in pDA12-*lpxL*$_{Bc}$. This plasmid was introduced into the conditional mutant strain YFM51 by triparental mating and exconjugants selected on LB agar plates containing 100 µg tetracycline ml^{-1}, 100 µg trimethoprim ml^{-1}, 200 µg ampicillin ml^{-1}, 20 µg polymyxin B ml^{-1} and 0.2% rhamnose.

Rhamnose depletion experiment

Conditional viability of mutants was assessed as described previously (Mohamed and Valvano, 2014, Ortega *et al.*, 2007). Conditional mutants were cultured in M9 minimal medium supplemented with 0.5% yeast extract, 100 µg trimethoprim ml^{-1} and 0.2% rhamnose and were left to grow overnight at 37°C. Bacterial pellets were washed three times with phosphate-buffered saline (PBS) and OD_{600} was adjusted to 1. Ten µl of 10^{-1} to 10^{-6} dilutions were inoculated onto LB agar plates supplemented with either 0.2% glucose or 0.2% rhamnose and incubated at 37°C for 24 h. The same procedure was used to

complement the loss of viability of YFM51 by expressing $MsbA_{Bc}$. The essentiality of $lpxM_{Bc}$ was further demonstrated by monitoring growth in liquid medium in a rhamnose depletion assay in which washed, rhamnose-depleted cells were diluted in M9 medium supplemented with 0.5% yeast extract and 0.2% glucose and aliquoted to 100-well honeycomb plate and monitored for growth for 20 h in a Bioscreen C (Oy Growth Curves Ab, Finland). Cultures were first diluted to OD_{600} of 0.1, allowed to grow for 4 h then further diluted to an OD_{600} of 0.005 in fresh M9 medium and grown as previously described (Mohamed and Valvano, 2014).

Microscopy

Samples of depleted cultures grown in the absence of rhamnose were taken at 2, 6, 16, and 20 h, placed on 0.8% agarose slides, covered with a coverslip and examined by phase-contrast and differential interference contrast microscopy using a Zeiss Axioimager 2. Bacteria were also stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit and images recorded by fluorescence microscopy. Images were processed by ImageJ on the Fiji platform (Schindelin *et al.*, 2012). Samples depleted for at lest 12 h were also examined by transmission electron microscopy after fixing with 2.5% glutaraldehyde and staining with 2% uranyl acetate and lead citrate, as previously described (Schmerk and Valvano, 2013). Grids were visualized with a Philips 410 transmission electron microscope at 60 kV in the Transmission Electron Microscopy Facility, Department of Microbiology and Immunology, University of Western Ontario.

Lipid A Extraction and Mass Spectrometry

Lipid A modifications were investigated by mass spectrometry (MS) using Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF). Cells were grown overnight in LB medium or for 16 h in M9 medium with 0.5% of yeast extract in the case of depletion experiments. For strains containing the P_{rha}::$LpxL_{Bc}$ insertion in the genome the medium was also supplemented with 100 µg trimethoprim ml^{-1} and either 0.2 % rhamnose or 0.2 %

glucose, as appropriate. Cultures were centrifuged (10,000 x*g*), washed twice with phosphate buffer (10 mM Na_2HPO_4, 1.7 mM KH_2PO_4) and freeze-dried. Lipid A was extracted from lyophilized cells and de-salted as previously described (El Hamidi *et al.*, 2005). Ten mg of the lyophilized bacterial cells were resuspended in 400 µl of a mixture of isobutyric acid:1 M ammonium hydroxide (5:3, v/v) and kept on a heat block for 2 h at 100°C with vortexing every 15 min. After centrifugation at 2000 x*g* for 15 min the supernatant was mixed with an equal volume of water and lyophilized overnight. Samples were then washed with methanol and lipid A was solubilized in 80 µl of chloroform:methanol:water (3:1.5:0.25, v/v). The lipid A suspension was desalted by adding a few grains of Dowex 50W-X8 (H^+) ion-exchange resin. A two-microliter aliquot of lipid A suspension was loaded on polished steel target, air dried and covered by 1 µl of 2,5-dihydroxybenzoic acid matrix (Sigma) dissolved in 0.1 M citric acid aqueous solution and allowed to air dry. The target was inserted in a Bruker Autoflex MALDI-TOF spectrometer. Data acquisition and analysis were performed using the Flex Analysis software.

Infection of murine RAW 264.7 macrophages

Murine RAW 264.7 cells were grown in Dulbecco's modified Eagle medium (DMEM) medium (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco), L-glutamine (2 mM; Gibco) and HEPES (10 mM; Gibco) at 37°C in a 95% humidified atmosphere with 5% CO2. For extraction of *B. cenocepacia* RNA, T75 Nunc flasks each containing a monolayer of macrophages (~10^7 cells) were infected with bacteria at a multiplicity of infection (MOI) of 200:1. To synchronize the infection the plates were centrifuged at 180 x*g* for 5min, and this was considered as time 0 h. After 1 h postinfection, extracellular bacteria were killed with 100 mg ml^{-1} gentamicin. After another 1 h, the DMEM media was replaced with fresh medium containing 10 µg ml^{-1} gentamicin. Incubation was continued for 6-8 h before additional processing of the samples for lipid A analyses or mRNA extraction.

Lipid A extraction from intracellular bacteria

Infected macrophages from 2 flasks, as described above, were pooled at 8 h postinfection, washed with PBS and resuspended in 800 µl of PBS. Two ml of methanol and 1 ml of chloroform were added and the suspension was mixed by vortexing and incubated for 30 min at room temperature with occasional vortexing. Pellets were then collected by centrifugation at 2000 x*g* for 15 min and resuspended in 4 ml of single-phase Bligh/Dyer system (chloroform:methanol:water, ratio 1:2:0.8 v/v respectively). Pellets were collected and resuspended in 400 µl of a mixture of isobutyric acid: 1 M ammonium hydroxide (5:3, v/v) and then processed as described above.

RNA extraction from intracellular bacteria and quantitative or real-time RT-PCR (qRT-PCR)

At 8 h postinfection, macrophages were lysed on ice for 30 min in 0.1% SDS, 1% acidic phenol, 19% ethanol in water as previously described (Eriksson *et al.*, 2003). Cells were then pooled from 6 flasks and pellets were collected by centrifugation and washed with a solution containing 0.1% acidic phenol, 19% ethanol in water, and then resuspended in 1 ml of tri-reagent (Ambion). RNA was prepared by traditional phenol-chloroform extraction and DNA was removed by treatment with DNAse (Roche) for 30 min at 37°C. Control RNA from *in vitro* bacteria was acquired by growing MH1K statically at 37°C in complete DMEM medium under 5% CO_2 to mimic conditions used for the infection experiments. RNA was then isolated as described above.

cDNA was obtained from 1 µg total RNA by using a commercial Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Invitrogen, UK) with random primers. Real-time PCR (RT-PCR) analyses were performed with a Mx3005p qPCR system (Agilent Technologies, UK). Hundred nanograms of cDNA were used as the template in a 20-µl reaction mixture containing KapaSYBR Fast qPCR mix (Kapa Biosystems) and primer mix. BCAS0175 and *gyrB* genes were amplified as housekeeping genes. The primers used are listed in Table S1. The thermocycling protocol was as follows: 95°C for 3 min for hot-start polymerase activation, followed by 45 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 30 s. SYBR green dye fluorescence was measured at 521 nm during the

annealing phase. Fold changes in gene expression were calculated using the Livak method ($\Delta\Delta$ C_T) (Livak and Schmittgen, 2001) with normalization to BCAS0175 and *gyrB* genes (Peeters *et al.*, 2010).

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Table 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics[a]	Source or reference
E. coli K-12		
DH5α	F$^-$ ϕ80 *lac*ZΔM15 *endA1 recA1 hsdR*17($r^-_K m^+_K$) *supE44 thi*-1Δ*gyrA*96 (Δ*lacZYA-arg*F)U169 *relA*1F$^-$ *mcrA* Δ*(mrr-hsdRMS-mcrBC)*	Invitrogen
GT115	ϕ*80lacZ*Δ*M15* Δ*lacX74 recA1 rpsL endA1* Δ*dcm uidA(*Δ*MluI)::pir-116* Δ*sbcC-sbcD*	Lab stock
W3110	F$^-$ I$^-$ rph-1 INV (*rrn*D-*rrn*E)1 *rph*-1	Lab stock
Δ*lpxL*	W3110, Δ*lpxL;* Kn^R	Lab stock
BN2	W3110, Δ*lpxM;* Kn^R	(Needham *et al.*, 2013)
B. cenocepacia		
K56-2	Clinical isolate, ET12 clone related to J2315	BCRRC[b]
MH1K	K56-2; Δ*amrABC* (BCAL1674-1676); Gm^S, Kn^S	(Hamad *et al.*, 2010)
MH1J	J2315; cystic fibrosis clinical isolate; Δ*amrABC* (BCAL1674-1676); Gm^S, Kn^S	(Hamad *et al.*, 2010)
MH76	K56-2; Δ*pagL* (BCAL0788)	This study
MV4179	MH1K; ΔO-antigen cluster (BCAL3119-BCAL3131)	(Hanuszkiewicz *et al.*, 2014)
YFM51	K56-2; P_{rha}::$lpxL_{Bc}$ (BCAL0508); Tp^R	This study
YFM52	K56-2; P_{rha}::$lpxL_{Bc}$ (BCAL0508); Tp^R pDA12-6xHis-$MsbA_{Bc}$;	This study
YFM53	K56-2; P_{rha}::$lpxL_{Bc}$ (BCAL0508); Tp^R pDA12	This study
Plasmids		
pDA12	Cloning vector, ori_{pBBR1}, Tet^R, *mob*$^+$, P_{dhfr}	(Aubert *et al.*, 2008)
pDAI-SceI-SacB	ori_{pBBR1}, Tet^R, P_{dhfr}, *mob*$^+$, expressing ISce-I, also expresses negative selection marker SacB	(Hamad *et al.*, 2010)
pGPISce-I	ori_{R6K}, *mob*$^+$, ΩTp^R , including an ISce-I restriction site	(Flannagan *et al.*, 2008)
pGPISce-I-2	pGPISce-I with additional unique cloning sites	(Hamad *et al.*, 2012).
pMH481	pGPISce-I; *pagL* deletion plasmid	This study
pRK2013	ori_{colE1}, RK2 derivative, Kn^R, *mob*$^+$, *tra*$^+$	(Figurski and Helinski, 1979)
pSC200	Cloning vector, ori_{pBBR1} rhaR, rhaS, *PrhaB*, Tp^R	(Cardona and Valvano, 2005)
pXO53	pSC200-$lpxL_{Bc}$	This study

pYM40	*msbA*$_{Bc}$ (BCAL2408) cloned in pDA12 with a C-terminal 6xHis tag, Tet^{R}	This study
pYM66	*lpxL*$_{Bc}$ (BCAL0508) cloned in pEXT20, Amp^{R}	This study

[a] Kn^{R}, kanamycin resistance; Gm^{S}, gentamicin sensitive; Kn^{S}, kanamycin sensitive; Tp^{R}, trimethoprim resistance, Tet^{R}, tetracycline resistance, Amp^{R}, ampicillin resistance.

[b]*Burkholderia cepacia* Research and Referral Repository for Canadian Cystic Fibrosis Clinics.

Table 2. List of observed *m/z* ion peaks by MALDI-TOF analysis of LPS samples from *B. cenocepacia* K56-2 parental and mutant strains used in this study

Observed ion (*m/z*)	Assignment
1154.6	Tri-acyl [2 GlcN + 14:0(3-OH) + 2 16:0(3-OH)], 1 P
1234.6	Tri-acyl [2 GlcN + 14:0(3-OH) + 2 16:0(3-OH)], 2 P
1285.7	Tri-acyl [2 GlcN + 14:0(3-OH) + 2 16:0(3-OH)], 1 Ara4N, 1P
1364.7	Tetra-acyl [2 GlcN + 14:0(3'-OH) + 2 16:0(3-OH) + 14:0], 1 P
1366.7	Tri-acyl [2 GlcN + 14:0(3-OH) + 2 16:0(3-OH)], 1 Ara4N, 2P
1444.7	Tetra-acyl [2 GlcN + 14:0(3'-OH) + 2 16:0(3-OH) + 14:0], 2 P
1460.9	Tetra-acyl [2 GlcN + 2 14:0(3-OH) + 2 16:0(3-OH)], 2 P
1495.9	Tetra-acyl [2 GlcN + 14:0(3'-OH) + 2 16:0(3-OH) + 14:0], 1 Ara4N, 1P
1497.1	Tri-acyl [2 GlcN + 14:0(3-OH) + 2 16:0(3-OH)], 2 Ara4N, 2P
1576.8	Tetra-acyl [2 GlcN + 14:0(3'-OH) + 2 16:0(3-OH) + 14:0], 1 Ara4N, 2P
1593.1	Tetra-acyl [2 GlcN + 2 14:0(3-OH) + 2 16:0(3-OH)], 1 Ara4N, 2 P
1671	Penta-acyl [2 GlcN + 14:0(3'-OH) + 2 16:0(3-OH) + 14:0(3-OH) + 14:0], 2 P
1707.9	Tetra-acyl [2 GlcN + 14:0(3'-OH) + 2 16:0(3-OH) + 14:0], 2 Ara4N, 2 P
1803.1	Penta-acyl [2 GlcN + 14:0(3'-OH) + 2 16:0(3-OH) + 14:0(3-OH) + 14:0], 1 Ara4N, 2P
1933.8	Penta-acyl [2 GlcN + 14:0(3'-OH) + 2 16:0(3-OH) + 14:0(3-OH) + 14:0], 2 Ara4N, 2P

Spectra corresponding to the ion peaks are shown in Fig. 4. The assignments were based on previously published data (Hamad *et al.*, 2012, Sturiale *et al.*, 2011). GlcN, glucosamine; P, phosphate; Ara4N, 4-L-amino-4-deoxyarabinose.

Figure Legends

Figure 1. Lipid A biosynthesis in *B. cenocepacia*. Lipid A biosynthetic enzymes in *B. cenocepacia* were identified using blast analysis with respect to their *E. coli* orthologues. The BCAL numbers correspond to the gene annotations of *B. cenocepacia* J2315, denoting genes in the larger chromosome.

A. The first 6 steps of the Raetz pathway, leading to the formation of lipid IV_A, are predicted to be identical as those in *E. coli*, with the exception that the two amide-linked acyl chains are 16:0(3-OH) residues (Silipo *et al.*, 2005).

B. Two Kdo residues are incorporated by the enzyme WaaA; the distal Kdo is modified into D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko) by KdoO (Chung and Raetz, 2011, Chung *et al.*, 2014). The doted circles indicate the Kdo groups modified by the Kdo hydroxylase; it is not clear if this step occurs before or after the addition of the 14:0(3-OH) acyl chain by LpxL. The incorporation of the secondary 14:0(3-OH) acyl chain (in grey) to the primary acyl chain at position 2', mediated by LpxL, is also indicated (dotted square).

Figure 2. $LpxL_{Bc}$ is required for the viability of *B. cenocepacia*.

A. Conditional lethal phenotype of strain K56-2 carrying the chromosomal P_{rha}::*lpxL*$_{Bc}$ transcriptional fusion that places the *lpxL* gene under the control of a rhamnose-inducible promoter. Bacterial culture dilutions were spotted on LB agar plates supplemented with 0.2% rhamnose (permissive condition) or 0.2% glucose (non-permissive condition).

B. Rhamnose depletion experiment in liquid medium of strain K56-2 carrying the P_{rha}::*lpxL*$_{Bc}$ transcriptional fusion. Growth was monitored every hour using a Bioscreen C instrument. Cultures were grown in M9 minimal medium with 0.2% rhamnose for 4 h (arrow) at which point bacteria were sedimented, washed with fresh medium with no rhamnose, and divided into two cultures with and without rhamnose. Solid black circles (permissive condition with 0.2% rhamnose); Open circles (non-permissive condition with 0.2% glucose). The figure is representative of three independent biological repeats with similar results.

C. The morphology of K56-2 P_{rha}::*lpxL* bacteria grown under permissive (0.2% rhamnose) and non-permissive (0.2% glucose) conditions was examined by differential interference contrast (DIC) using oil-immersion objective lens in a Zeiss Axioscope II microscope (Magnification: 1,000 X). Bacteria were also stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit. Images recorded in the green fluorescent channel, which were stained with Syto9 are shown. Insets in each panel show additional detail, especially the fluorescently stained disorganized nucleoids (arrowheads) and the constrictions indicating defects in bacterial cell division (arrows).

Figure 3. Ultrastructure of the K56-2(P_{rha}::*lpxL*) bacterial cells. Bacteria were examined by transmission electron microscopy after fixation with 2.5% glutaraldehyde and staining with 2% uranyl acetate and lead citrate. Bar, 0.5 µm

A. Bacteria grown in 0.2% rhamnose (permissive condition).

B-F. Bacteria initially grown in rhamnose were depleted for 12 h in 0.2% glucose. Empty sacculi (black arrows) and accumulation of membranous structures (white arrows) are shown.

Figure 4. Characterization of the lipid A in *B. cenocepacia* strain K56-2 carrying the P_{rha}::*lpxL* promoter fusion under permissive and non-permissive conditions and the isogenic Δ*pagL* mutant.

A. Negative ion MALDI-TOF mass spectra of lipid A isolated from K56-2 P_{rha}::*lpxL* grown under permissive (rhamnose) and non-permissive (glucose) conditions. Ara4N, 4-L-amino-4-deoxyarabinose.

B. The deduced bisphosphorylated lipid A structures produced by the K56-2 P_{rha}::*lpxL* strain under permissive (rhamnose) and non-permissive (glucose) conditions are shown as a reference. The structures with one phosphate and with one or two Ara4N molecules are not represented. The LpxL-mediated acyl chain addition is shown in grey (dotted rectangles). The 3' and 3 positions of the lipid A where the β-hydroxymyristate (3-OH-C14:0) is attached by LpxA (see Fig. 1) and removed from the 3-position by the PagL deacylase (see panels C and D) are indicated.

C. Negative ion MALDI-TOF mass spectra of lipid A isolated from K56-2 and the Δ*pagL* mutant. Ara4N, 4-L-amino-4-deoxyarabinose.

D. Deduced bisphosphorylated lipid A structures produced by K56-2 and the Δ*pagL* mutant shown for reference. Alternative structures are not represented. The LpxL-mediated acyl chain addition is shown in grey. The 3' and 3 positions of the lipid A where the β-hydroxymyristate (3-OH-C14:0) is attached by LpxA (see Fig. 1) and removed from the 3-position by the PagL deacylase are indicated.

Figure 5. Overexpression of MsbA rescues growth of K56-2 P_{rha}::$lpxL_{Bc}$ under the non-permissive condition.

A. Growth of the conditional mutant K56-2 P_{rha}::$lpxL_{Bc}$ strain with vector control (pDA12) (left panel) and with pDA12-$msbA_{Bc}$ (right panel). Bacteria were grown overnight with 0.2% rhamnose, serially diluted and plated on LB supplemented with 0.2% glucose (non-permissive condition).

B. Phase contrast microscopy of K56-2 P_{rha}::*lpxL*$_{Bc}$ bacterial cells containing the pDA12 vector control (left panel) and K56-2 P_{rha}::*lpxL*$_{Bc}$ cells overexpressing MsbA$_{Bc}$ (right panel) under the non-permissive condition.

C. Depletion experiments using K56-2 P_{rha}::*lpxL*$_{Bc}$ carrying either pDA12 (vector control) or pDA12-*msbA*$_{Bc}$. The figure is representative of three independent biological repeats with similar results.

D. Negative ion MALDI-TOF mass spectrum of lipid A extracted from K56-2 P_{rha}::*lpxL*$_{Bc}$ + MsbA$_{Bc}$ under the non-permissive condition.

Figure 6. Complementation of *E. coli* Δ*lpxL* by *B. cenocepacia* LpxL$_{Bc}$. Negative ion MALDI-TOF mass spectra of lipid A isolated from the parental *E. coli* K12 strain W3110 and the W3110 Δ*lpxL*$_{Ec}$ and Δ*lpxM*$_{Ec}$ mutans alone or complemented by the introduction of a plasmid encoding the *B. cenocepacia* LpxL$_{Bc}$ protein. The insert indicates the structural representation of the lipid A structure deduced from the MALDI-TOF mass spectrum in the Δ*lpxL*$_{Ec}$ mutant complemented with LpxL$_{Bc}$, which shows hexa-acylated lipid A containing a myristoyl (C14:0) group (dotted-line rectangle) incorporated by LpxL$_{Bc}$. The myristoyl group incorporated by the *E. coli* LpxM is also indicated.

Figure 7. Characterization of *B. cenocepacia* lipid A produced by intracellular bacteria within macrophages. Negative ion MALDI-TOF mass spectra of lipid A purified from MH1J and MH1K derivatives of strains J2315 and K56-2, respectively. These derivatives contain a deletion of an antibiotic efflux pump that allows monitoring bacterial intracellular survival by eliminating extracellular bacteria upon incubation with kanamycin (Hamad *et al.*, 2010). Intracellular bacteria were collected 8 h post-infection and processed to obtain lipid A for analysis as described in *Experimental Procedures*

A. Control lipid A from MH1J grown in LB.

B. Mass spectrum profile of non-infected RAW264.7 murine macrophages as a negative control.

C. Lipid A from intracellular MH1J in RAW264.7 murine macrophages

D. Lipid A from intracellular MH1K ΔBCAL3119-3131 (ΔO-antigen) in RAW264.7 murine macrophages. The loss of O-antigen facilitates *B. cenocepacia* intracellular infection in macrophages (Saldías *et al.*, 2009).

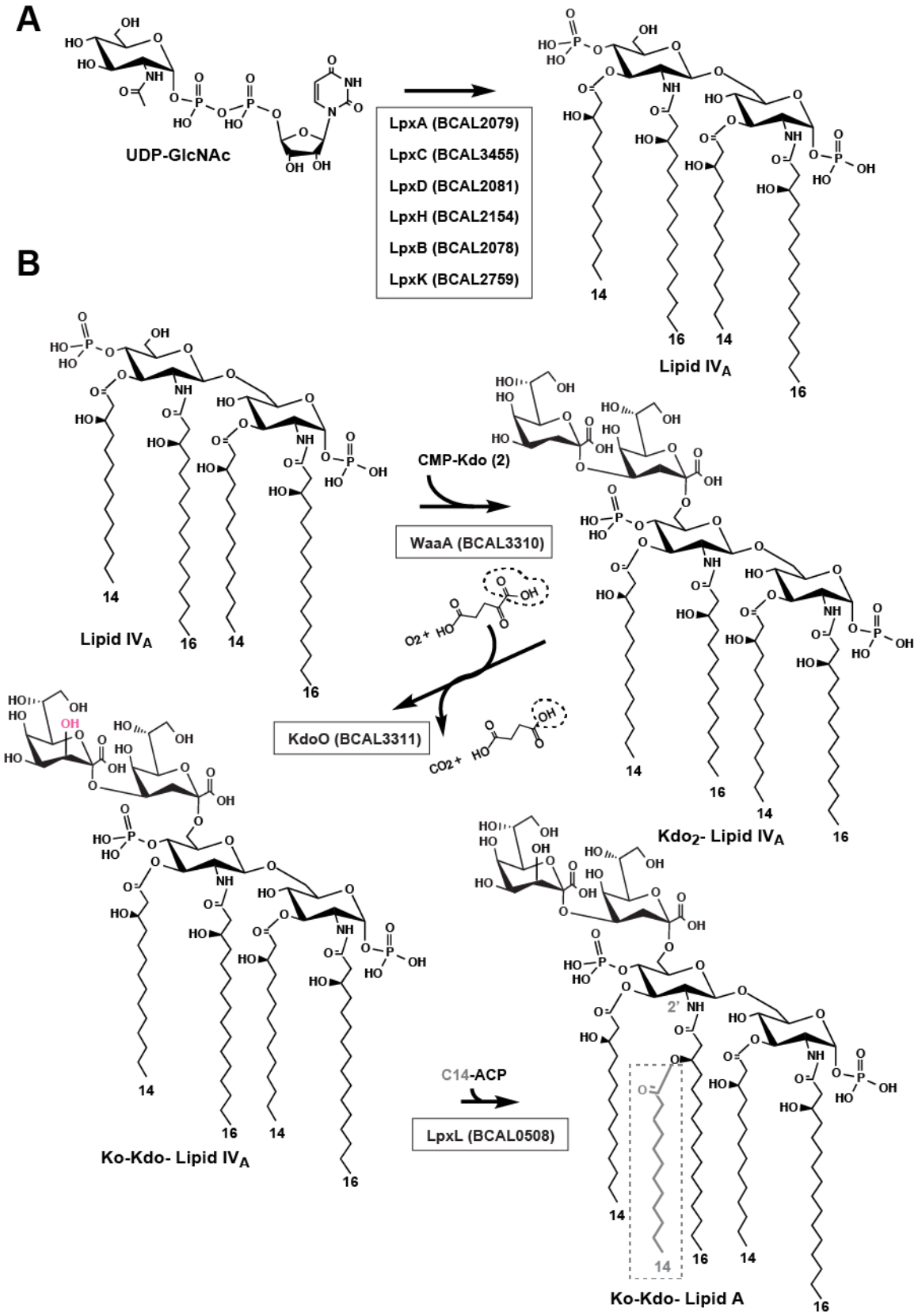

A
UDP-GlcNAc
LpxA (BCAL2079)
LpxC (BCAL3455)
LpxD (BCAL2081)
LpxH (BCAL2154)
LpxB (BCAL2078)
LpxK (BCAL2759)
Lipid IVA
B
CMP-Kdo (2)
WaaA (BCAL3310)
KdoO (BCAL3311)
Kdo2- Lipid IVA
Ko-Kdo- Lipid IVA
C14-ACP
LpxL (BCAL0508)
Ko-Kdo- Lipid A

fig 1

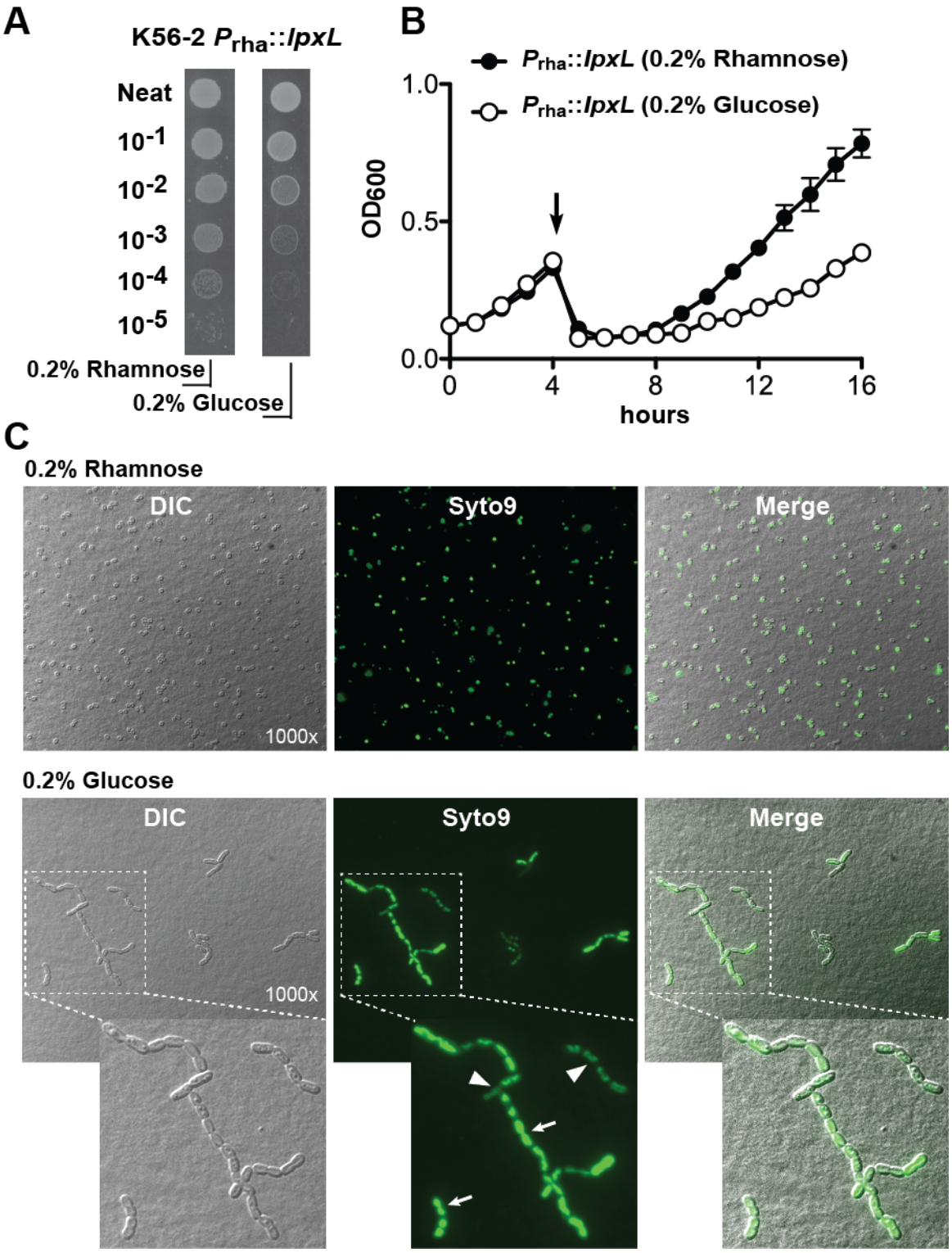

A
K56-2 P_{rha}::*lpxL*
Neat
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
0.2% Rhamnose
0.2% Glucose
B
P_{rha}::*lpxL* (0.2% Rhamnose)
P_{rha}::*lpxL* (0.2% Glucose)
OD600
1.0
0.5
0.0
0
4
8
12
16
hours
C
0.2% Rhamnose
DIC
Syto9
Merge
1000x
0.2% Glucose
DIC
Syto9
Merge
1000x

fig 2

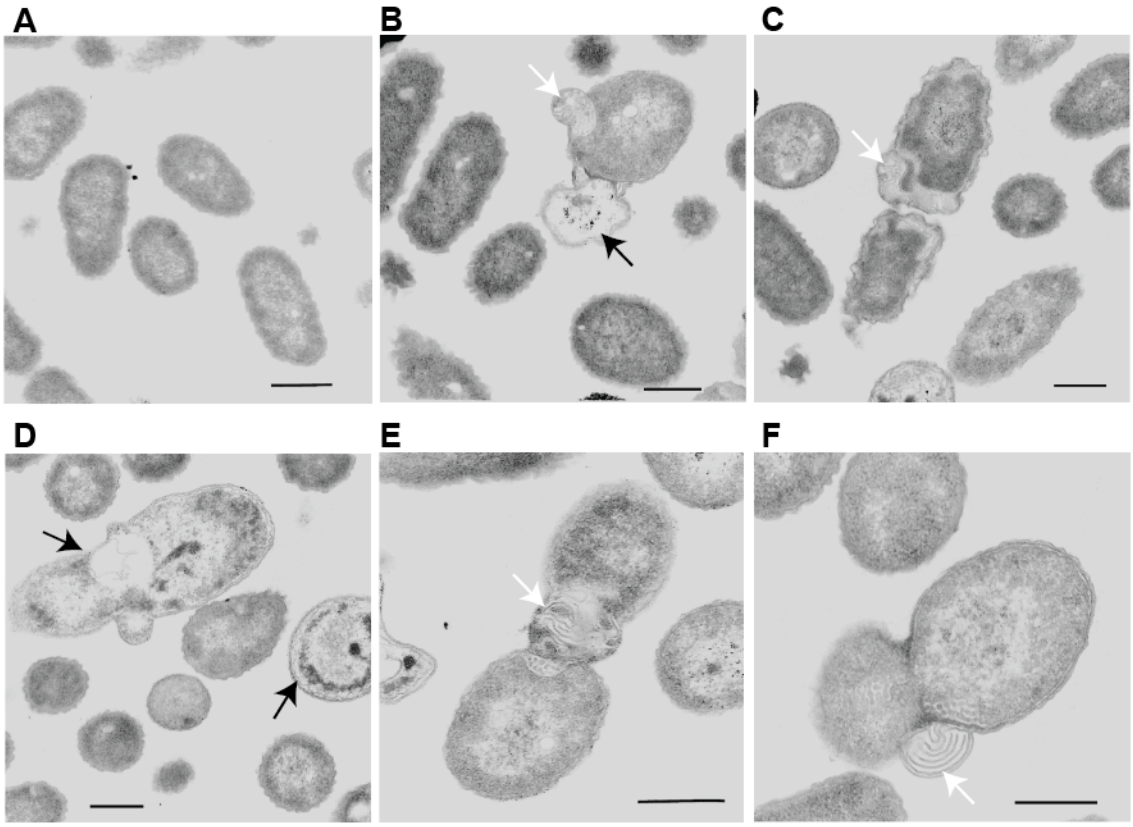


fig 3

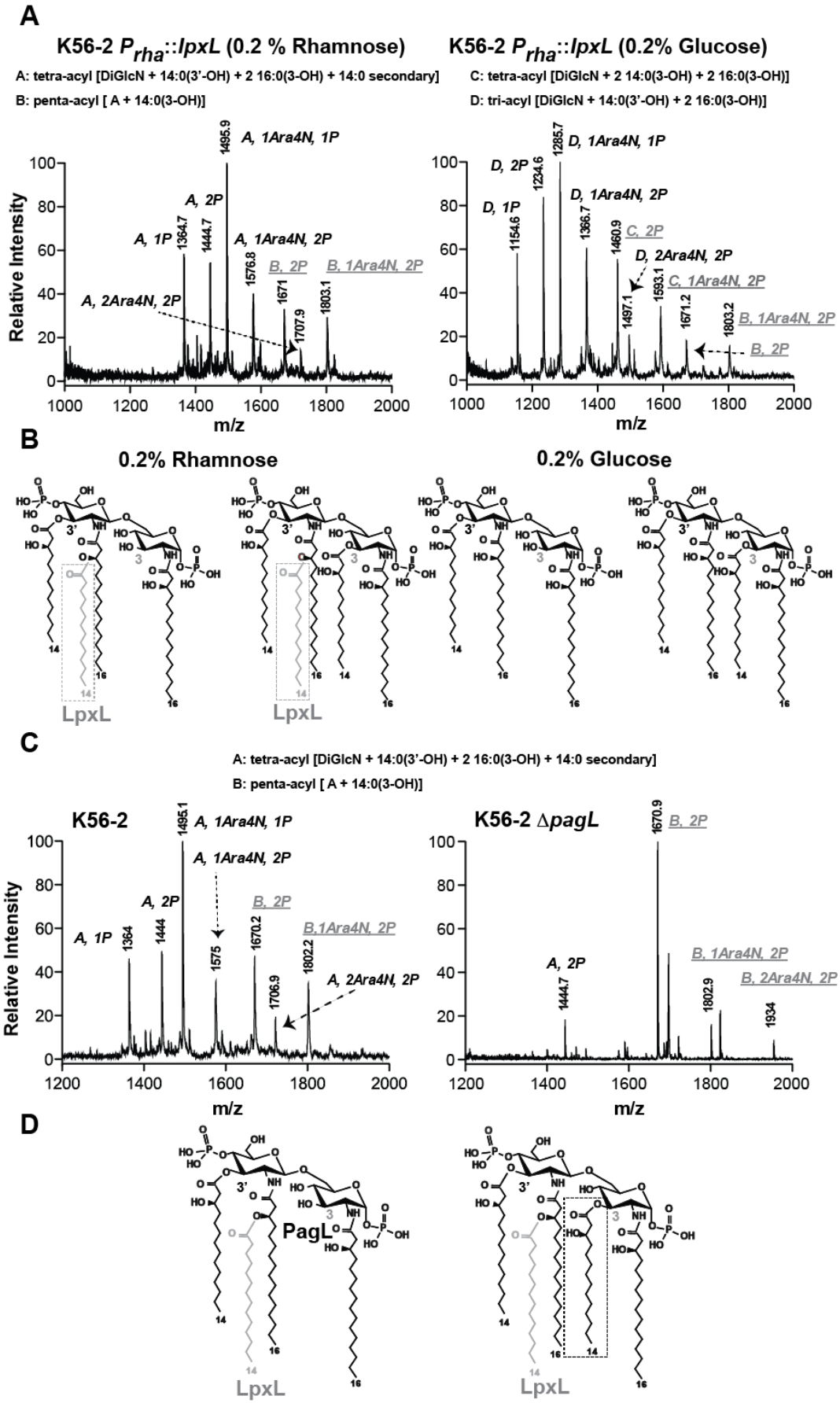

A
K56-2 P_{rha}::*lpxL* (0.2 % Rhamnose)
K56-2 P_{rha}::*lpxL* (0.2% Glucose)
A: tetra-acyl [DiGlcN + 14:0(3'-OH) + 2 16:0(3-OH) + 14:0 secondary]
B: penta-acyl [A + 14:0(3-OH)]
C: tetra-acyl [DiGlcN + 2 14:0(3-OH) + 2 16:0(3-OH)]
D: tri-acyl [DiGlcN + 14:0(3'-OH) + 2 16:0(3-OH)]
Relative Intensity
m/z
A, 1Ara4N, 1P
A, 2P
A, 1P
A, 1Ara4N, 2P
A, 2Ara4N, 2P
B, 2P
B, 1Ara4N, 2P
D, 2P
D, 1P
D, 1Ara4N, 1P
D, 1Ara4N, 2P
C, 2P
D, 2Ara4N, 2P
C, 1Ara4N, 2P
B
0.2% Rhamnose
0.2% Glucose
LpxL
C
A: tetra-acyl [DiGlcN + 14:0(3'-OH) + 2 16:0(3-OH) + 14:0 secondary]
B: penta-acyl [A + 14:0(3-OH)]
K56-2
K56-2 ΔpagL
A, 1Ara4N, 1P
A, 1Ara4N, 2P
A, 2P
A, 1P
B, 2P
B,1Ara4N, 2P
A, 2Ara4N, 2P
B, 1Ara4N, 2P
B, 2Ara4N, 2P
D
PagL
LpxL

fig 4

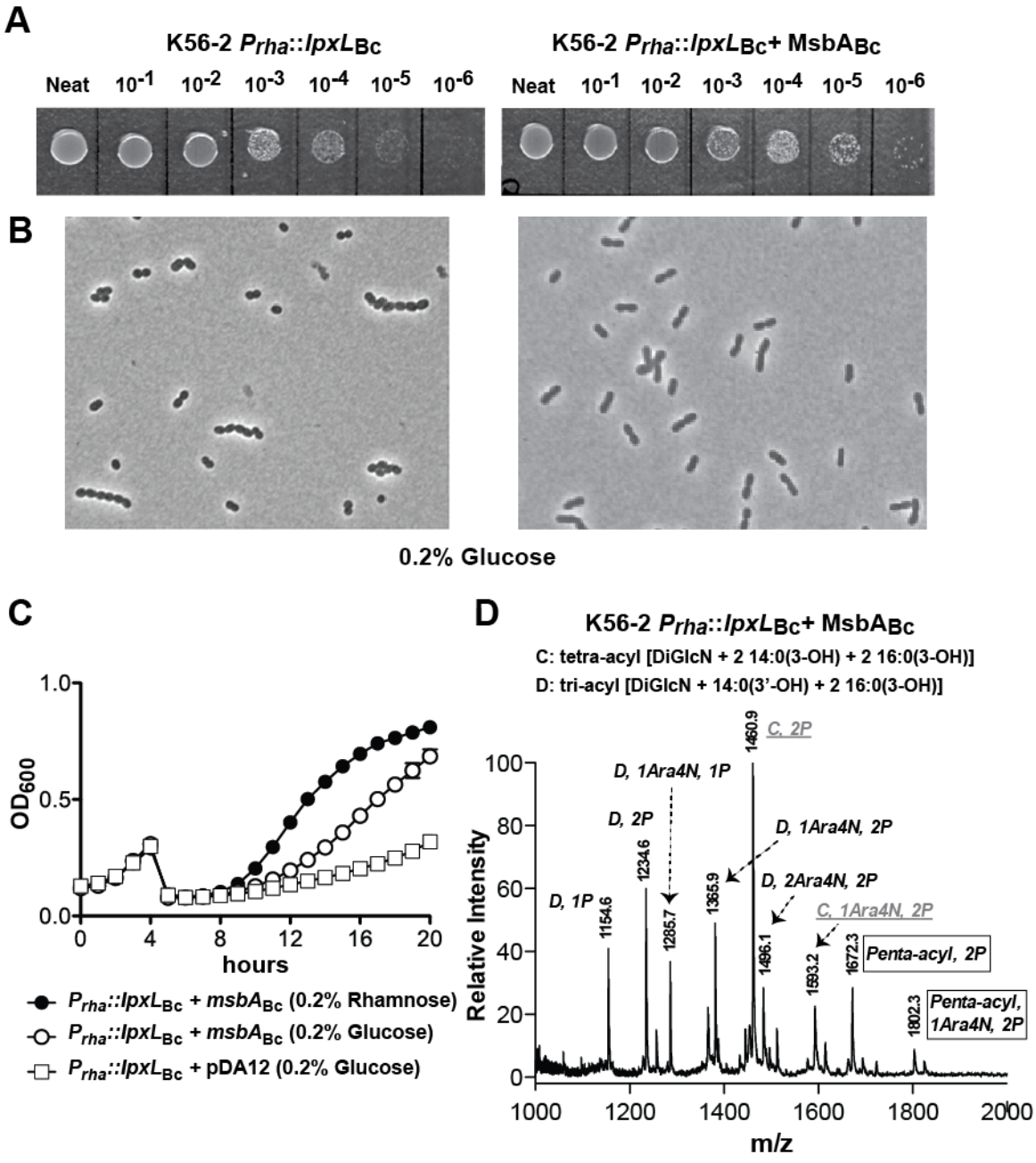

A
K56-2 Prha::lpxLBc
K56-2 Prha::lpxLBc+ MsbABc
Neat 10-1 10-2 10-3 10-4 10-5 10-6
B
0.2% Glucose
C
OD600
hours
Prha::lpxLBc + msbABc (0.2% Rhamnose)
Prha::lpxLBc + msbABc (0.2% Glucose)
Prha::lpxLBc + pDA12 (0.2% Glucose)
D
K56-2 Prha::lpxLBc+ MsbABc
C: tetra-acyl [DiGlcN + 2 14:0(3-OH) + 2 16:0(3-OH)]
D: tri-acyl [DiGlcN + 14:0(3'-OH) + 2 16:0(3-OH)]
Relative Intensity
m/z
D, 1P
D, 2P
D, 1Ara4N, 1P
D, 1Ara4N, 2P
D, 2Ara4N, 2P
C, 2P
C, 1Ara4N, 2P
Penta-acyl, 2P
Penta-acyl, 1Ara4N, 2P
1154.6
1234.6
1285.7
1365.9
1460.9
1496.1
1593.2
1672.3
1802.3

fig 5

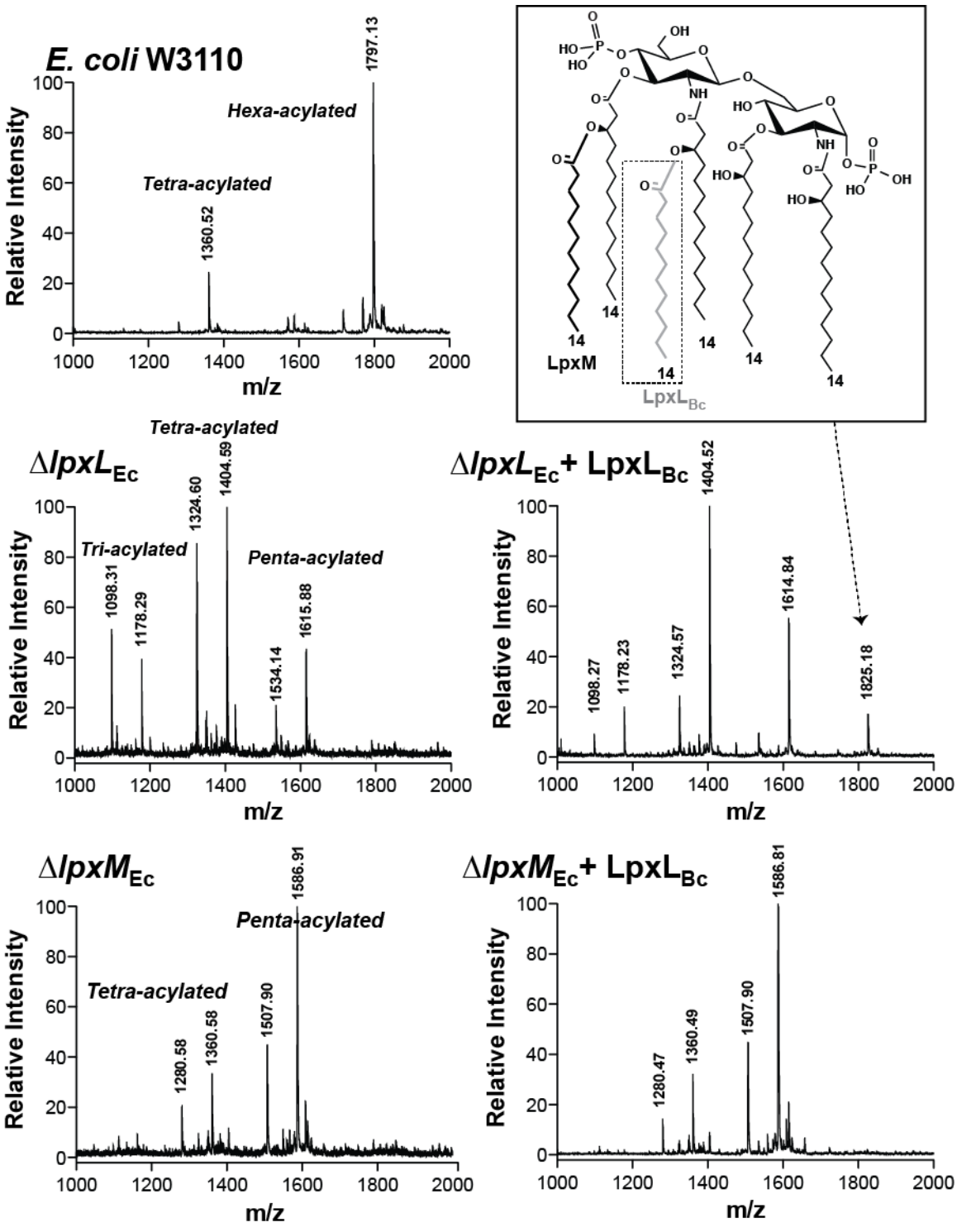
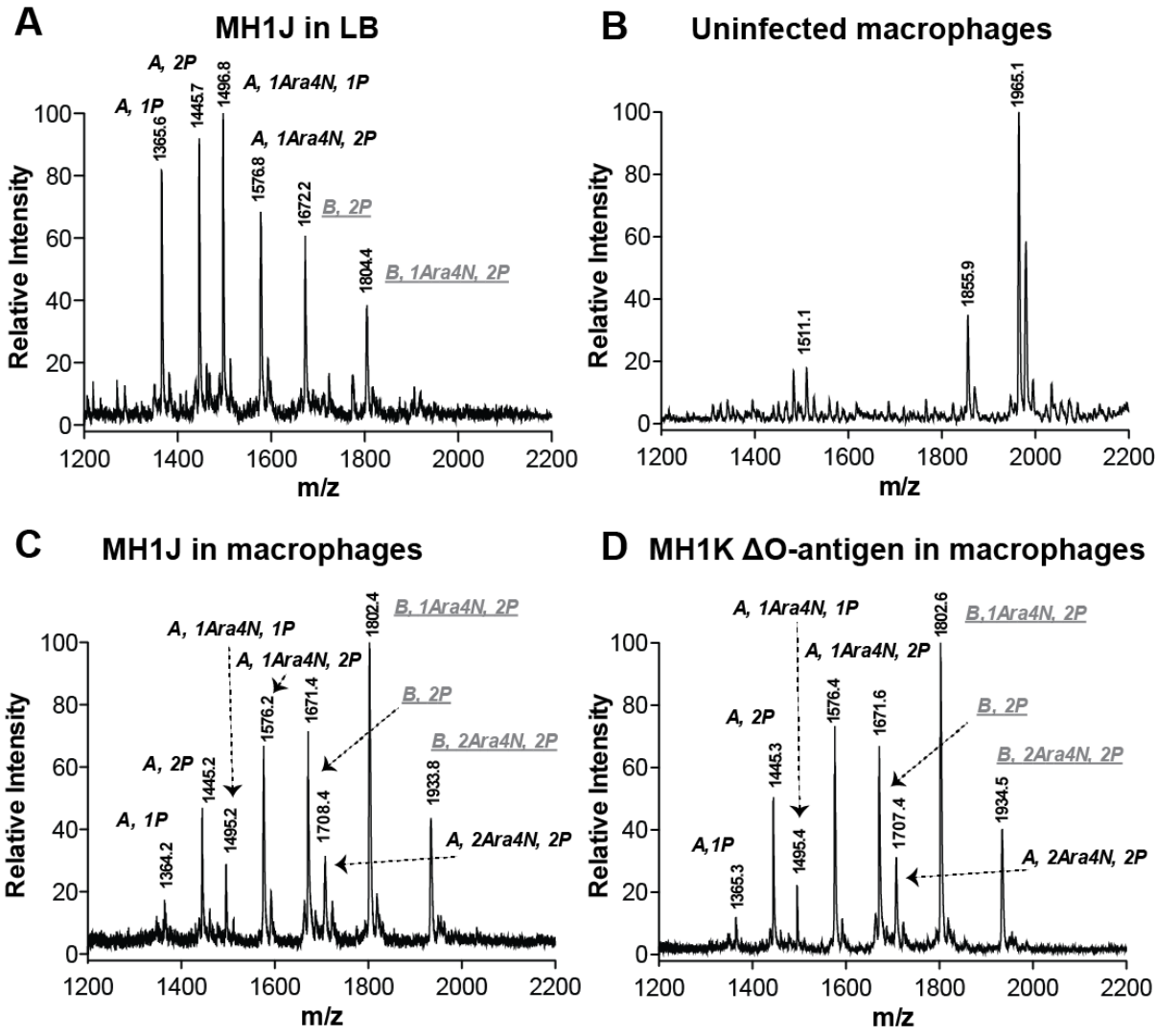

E. coli W3110
Hexa-acylated
Tetra-acylated
1797.13
1360.52
Relative Intensity
m/z
LpxM
LpxL_Bc
14
Tetra-acylated
ΔlpxL_Ec
Tri-acylated
Penta-acylated
1324.60
1404.59
1098.31
1178.29
1534.14
1615.88
ΔlpxL_Ec+ LpxL_Bc
1404.52
1614.84
1098.27
1178.23
1324.57
1825.18
ΔlpxM_Ec
1586.91
Penta-acylated
Tetra-acylated
1507.90
1360.58
1280.58
ΔlpxM_Ec+ LpxL_Bc
1586.81
1507.90
1360.49
1280.47

fig 6



A: tetra-acyl [DiGlcN + 14:0(3'-OH) + 2 16:0(3-OH) + 14:0 secondary]

B: penta-acyl [A + 14:0(3-OH)]

fig 7

Table S1. Primers

Primer	Oligonucleotide sequence (5'- 3')	Restriction enzyme
Cloning of *lpxL*$_{Bc}$ (BCAL0508)		
Q868	AAAACCCGGGCGGAGATTCCGCATGCTAGGC	*Sma*I
Q867	AAAATCTAGATCAGTACAGGCTCGGCTCG	*Xba*I
Cloning of *msbA* (BCAL2408)		
Q714	TTTTTTCATATGGATTTTCGATTCAAATTCCTTGA	*Nde*I
Q715	TTTTTTTCTAGATCAGTGGTGGTGGTGGTGGTGCCCGGCCTCCTGCTGGAACTGAATC	*Xba*I
Mutagenesis of *pagL* (BCAL0788)		
4740	AGCGCTAGCAATATTCTACGCGGGCTCGAAAGGCGCGGTC	*Ssp*I
4741	GCATGAATCGATGGACGTGCGTCGCCGGCTGGGCGC	*Cla*I
4728	AGCTAGATCGATGAGTGGAAGCTGTTCTGGACGGG	*Cla*I
4729	CAGCTATCTAGAACGCC GCGCGCGTTTGCCGCCGATTC	*Xba*I
qRT-PCR primers		
BCAS0175		
Q800 Forward	ATGGCCAGTTCGCTCATCA	
Q801 Reverse	ACGCGATGTCGATACTCGAAT	
gyrB		
Q818 Forward	GTTCCAGTCCATCGCGACTT	
Q819 Reverse	GGGCTTCGTCGAATTCATCA	
***lpxL* (BCAL0508)**		
Q816 Forward	CTATCGTCTGAGGGTGTTCAAG	
Q817 Reverse	TCGAGGAATTCGTTCATCCG	
***pagL* (BCAL0788)**		
BCAL0788 Forward	TTCCGCTTCATCAAGAGCAG	
BCAL0788 Reverse	CGAGAAATTGTCCGAGATCGTC	